

Immunization with *Staphylococcus aureus* Lysate Incorporated into Microspheres¹

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ABSTRACT

Antibiotics are of limited value against *Staphylococcus aureus* due to development of resistant strains, scar tissue formation, and blockage of ducts due to inflammation. Though macrophages are the predominant cell type in the mammary gland, they are primarily scavenger cells and are not effective against bacteria entering the gland. Neutrophil phagocytosis is the bovine's primary defense against *S. aureus* mastitis. Attempts to develop vaccines that enhance neutrophil phagocytosis by stimulating production of opsonizing antibodies to *S. aureus* have met with limited success because of the low immunogenicity of the exopolysaccharide capsule surrounding *S. aureus*. *Staphylococcus aureus* can also adhere to and penetrate epithelial tissue. This study was conducted to determine whether lysates of *S. aureus* encapsulated in biodegradable microspheres would increase the production of opsonizing antibodies to capsule and block adherence. Four groups of four cows each were injected with 1 ml of the respective treatment in the area of the supramammary lymph node and 1 ml in the hip muscle. The treatments were: lysate in NaCl, lysate in Freund's incomplete adjuvant (FICA), lysate in microspheres in NaCl, and lysate in microspheres in FICA. Antigen in microspheres produced a similar antibody response to antigen emulsified in FICA, but to a lesser magnitude. Antigen in microspheres produced antibodies that were more opsonic for neutrophils at 20 and 52 wk postimmunization and inhibited *S. aureus* adherence to mammary epithelium. Ability to control antigen release and presentation, and the benefit of a single injection for long-term immunity using microspheres warrants additional studies.

(**Key words:** microsphere, *Staphylococcus aureus*, lysate, phagocytosis)

Abbreviation key: Cp = *Staphylococcus aureus* Smith Compact (unencapsulated variant of Df), ddH₂O = distilled deionized water, Df = *Staphylococcus aureus* Smith Diffuse, Lys/FICA = Df lysate emulsified in FICA, Lys/NaCl = Df lysate in NaCl, Lys-S/FICA = Df lysate encapsulated in microspheres and emulsified in FICA, Lys-S/NaCl = Df lysate encapsulated in microspheres suspended in NaCl, RT = room temperature.

INTRODUCTION

Staphylococcus aureus is a major cause of mastitis in cattle (Foster, 1986; Watts, 1988; Watts et al., 1986). Limited effectiveness of antibiotics has led researchers to study the cows' natural defense mechanisms. This research has shown that neutrophil phagocytosis is the most effective defense against *S. aureus* infection in the bovine mammary gland (Paape et al., 1981). But, neutrophils in milk are less phagocytic because of ingestion of milk fat globules and casein and a deficiency of opsonins. Therefore, high concentrations of neutrophils (9×10^5 neutrophils/ml) are needed to prevent intramammary infections (Sears et al., 1990). Because this concentration exceeds the number of neutrophils in the healthy bovine gland, emphasis has been placed on increasing neutrophil efficiency by increasing opsonizing antibodies in lacteal secretions, thereby decreasing the number of neutrophils needed for protection of the gland against infection.

Although some progress has been made, no protective *S. aureus* vaccine has been developed. A major obstacle to production of a *S. aureus* vaccine is the extracellular polysaccharide capsule, which forms when the organism enters the gland (Caputy and Costerton, 1984; Norcross and Opdebeeck, 1983; Wilkinson, 1983). The capsule allows for antibodies directed against the cell wall to penetrate but masks recognition of the antibody by neutrophils (Hill, 1981; Peterson et al., 1978; Wilkinson, 1983) and prevents activation of complement (Blo-

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bel et al., 1980; Marques et al., 1992; Peterson et al., 1978). Therefore, attempts have been made to produce antibodies to bacterial antigens that would not be masked by capsule, thus allowing for effective phagocytosis by neutrophils. Polysaccharides are weak immunogens and are T-cell independent, therefore are poor immunizing agents (Poolman, 1990; Stein, 1992). However, when antibodies to capsule are produced, they serve as effective opsonins for neutrophils (Guidry et al., 1991; Karakawa et al., 1988; Lee et al., 1987).

Instability of the capsule has been a major obstacle to studying the effect of capsule on *S. aureus* virulence and on production of opsonizing antibodies to encapsulated *S. aureus*. From 94 to 100% of fresh isolates from cases of *S. aureus* mastitis are encapsulated, but fail to express capsule when cultured in vitro (Norcross and Opdebeeck, 1983; Rather et al., 1986). This obstacle was circumvented in the current study by using the Smith Diffuse strain of *S. aureus* (Df), which has a rigid stable capsule regardless of growth conditions.

Another aspect of this study was to produce a vaccine that would give a sustained immune response with a single injection of the vaccine. A previous report demonstrated the feasibility of obtaining a sustained immune response to polysaccharides, conjugated to a carrier protein, incorporated in poly(DL-lactide-co-glycolide) microspheres (O'Brien et al., 1996). In the current study, a lysate of Df was encapsulated in poly(DL-lactide-co-glycolide) microspheres in an effort to obtain effective opsonizing antibodies to both cell wall and capsule.

MATERIALS AND METHODS

Preparation of *S. aureus* Df Lysate

Staphylococcus aureus Df was grown overnight in trypticase soy broth, 3% formalin-killed, washed twice with PBS, and stored at 4°C. One milliliter of Df (6.68×10^{10} /ml) was suspended in 8 ml 0.05M Tris pH 7.5. Thirty-five units of lysostaphin (Sigma Chemical Co., St. Louis, MO) in 0.05M Tris pH 7.5 buffer containing 0.145M NaCl was added. The mixture was incubated for 6 h in a 37°C water bath with shaking (100 rpm). The reaction was monitored by periodically measuring the absorbance at OD₅₈₀. The lysostaphin was inactivated by placing the tube in a 75°C water bath for 15 min. The mixture was cooled then filtered through a 0.45- μ m filter to remove intact bacteria. An additional 1 ml of Df and lysostaphin was added to this filtrate, and the process repeated. The Df lysate was assayed for the presence of major *S. aureus* cell wall components (protein A, teichoic acid, and peptidoglycan) and capsule using the ELISA. A monoclonal antibody to protein A and antisera to peptidoglycan, teichoic acid, and Df capsule that had been absorbed with *S. aureus* Smith

Compact (unencapsulated variant of Df) (Cp) to remove cell wall antibodies, reacted with Df lysate preparation, indicating the presence of cell wall and capsular antigens (data not shown).

Production of Microspheres (<10 μ m)

Five hundred microliters of Df lysate in distilled deionized water (ddH₂O) was emulsified with a 6% copolymer solution [300 mg of poly(DL-lactide-co-glycolide) (Sigma Chemical Co.), 50:50 lactide:glycolide ratio, dissolved in 5 ml of dichloromethane (Aldrich Chemical Co., Milwaukee, WI)], with an Ultra-Turrax T25 homogenizer (Tekmar, Cincinnati, OH) at 12,000 rpm, for 1 min. The emulsion was added to 10 ml of 5% polyvinyl alcohol (88% hydrolyzed, 13,000–23,000 MW; Aldrich Chemical Co.) and homogenized at 12,000 rpm for 1 min to produce a water-oil-water emulsion. The resulting mixture was stirred overnight at room temperature (RT) to allow solvent evaporation. The microspheres were collected by centrifugation at $1000 \times g$ for 10 min, washed twice with ddH₂O, and freeze-dried. Encapsulation of the small microspheres was 63%.

Production of Microspheres (>10 μ m)

One milliliter of Df lysate in ddH₂O was emulsified with a 10% copolymer solution [1.0 g of poly(DL-lactide-co-glycolide) with an 75:25 lactide:glycolide ratio, dissolved in 10 ml of dichloromethane] with an Ultra-Turrax T25 homogenizer at 12,000 rpm for 1 min. The emulsion was added dropwise to 100 ml of a 3% aqueous solution of polyvinyl alcohol at RT with constant stirring (approximately 200 rpm). Microspheres formed immediately, but were allowed to stir overnight at RT to allow solvent evaporation. The microspheres were collected by centrifugation at $1000 \times g$ for 10 min and washed twice with ddH₂O, then freeze-dried. Encapsulation of the large microspheres was 53%.

Determining Encapsulation Efficiency of Small Microspheres (<10 μ m)

Antigen content of the microspheres was determined as described by Hora et al. (1990). Microspheres (10 to 15 mg) were dissolved in 3 ml of 0.1M NaOH containing 5% SDS, pH 7.4, and rotating overnight. After centrifugation, the aqueous phase was removed and filtered with a 0.45- μ m filter. Protein content of the aqueous phase was determined by absorbance at OD₂₈₀ with protein A dissolved in 0.1M NaOH containing 5% SDS, pH 7.4, as the standard curve. The procedure was performed in duplicate.

Determining Encapsulation Efficiency of Large Microspheres (>10 μm)

Antigen content of the large microspheres was determined by measuring the amount of unencapsulated antigen. After the first centrifugation to pellet the microspheres, the supernatant was collected and concentrated to 10 ml (10 \times) with a pressure dialyzer (Amicon Corp., Lexington, MA). The protein content of the supernatant was determined by the micro bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Protein A in 3% polyvinyl alcohol that had been concentrated 10 \times , similar to the supernatants, served as the standard.

Immunization of Cows with *S. aureus* Lysate Encapsulated in Microspheres

Healthy nonlactating Holstein cows were screened for low sera titers to *S. aureus* CP and Df. Cows were randomly divided into four groups of four each. Cows in the respective groups were immunized with 500 μg of Smith Df lysate in 0.85% NaCl (**Lys/NaCl**), 500 μg of Smith Df emulsified in Freund's incomplete adjuvant (**Lys/FICA**), 500 μg of Df lysate encapsulated in microspheres suspended in 0.85% NaCl (**Lys-S/NaCl**), and 500 μg of Df lysate encapsulated in microspheres emulsified in FICA (**Lys-S/FICA**). The cows were immunized at drying off by injecting 1 ml of the vaccine intramuscularly and 1 ml in the area of the supramammary lymph node. The microspheres used for immunization were composed of small (<10 μm) and large (>10 μm) microspheres. Half of the injected antigen (250 μg) was encapsulated in small microspheres composed of 50% lactic acid and 50% glycolic acid. The other half of the antigen was encapsulated in large microspheres composed of 75% lactic acid and 25% glycolic acid. The cows were bled via the tail vein at 0, 2, 4, 7, 10, 13, 16, 20, and 52 wk. The blood was allowed to clot and the serum was collected via centrifugation and stored at -20°C.

ELISA to Determine Bovine Anti-Cell Wall Serum Titers

Wells of Immulon 2 round-bottom plates (Dynatech, Chantilly, VA) were coated with Cp (2×10^8 cells/ml of 0.01M PBS, 0.74% NaCl, pH 6.8) and incubated overnight at RT. After washing twice with 0.85% NaCl containing 0.05% Tween 20, 100 μl of bovine sera that had been incubated with soluble protein A (200 μg /ml, overnight at 4°C; Sigma Chemical Co.) to block Fc binding, and diluted in PBS containing 0.05% Tween 20 (diluent) were added and incubated for 2 h. The plates were washed three times and goat anti-bovine IgG labeled with alkaline phosphatase (Kirkegaard and

Perry, Gaithersburg, MD, diluted 1/500 in diluent) was added and incubated for 2 h at RT. Substrate was added and the plates read at OD₄₀₅ on a Bio-Tek plate reader (Bio-Tek, Winooski, VT).

Isolation of *S. aureus* Capsule

Staphylococcus aureus Smith Diffuse large clearing (very large flaccid capsule) was grown on trypticase soy agar. The organisms were removed by washing the agar plates with PBS, 3% formalin-killed for 18 h, washed, and suspended in PBS (5% wet wt/vol). The bacterial suspension was mixed in a Waring blender at 21,500 rpm for 3 min at 4°C then cooled on ice for 3 min. This cycle was repeated four times. Organisms were removed via centrifugation at 4500 $\times g$ for 10 min. Supernatants were pooled and saved as the capsule preparation. The capsule preparation was assayed for presence of the major *S. aureus* cell wall components (protein A, teichoic acid, and peptidoglycan) using the ELISA.

ELISA to Determine Bovine Anticapsular Serum Titers

Wells of Immulon 2 round-bottom plates were coated with poly-L-lysine (0.01 mg/ml of PBS; Sigma Chemical Co.) for 30 min at RT. The wells were washed three times and 100 μl of *S. aureus* capsule (1 μg /ml in PBS) was added and incubated overnight at RT. After washing, gamma globulin-free horse serum in diluent was used to block unbound sites. Bovine sera that had been diluted in diluent was added and incubated for 2 h. The plates were washed three times, and 100 μl of goat anti-bovine IgG labeled with alkaline phosphatase (Kirkegaard and Perry), diluted 1/500 in diluent, was added to the wells and incubated for 2 h. After washing, 100 μl substrate (*p*-nitrophenyl phosphate disodium; Sigma Chemical Co.) was added, and the plates were read at OD₄₀₅ on a Bio-Tek plate reader.

ELISA to Determine Bovine Anti-Cell Wall and Anticapsular Antibody Isotype

Wells of Immulon 2 round-bottom plates were coated with Cp or capsule. Bovine serum samples were added and incubated as above. After washing, 100 μl rabbit anti-bovine IgG₁, IgG₂ polyclonal sera, or mouse anti-bovine IgM monoclonal antibody, diluted in PBS containing 0.2% Tween 20 was added to the wells and incubated for 2 h. Then sheep anti-rabbit IgG labeled with alkaline phosphatase or goat anti-mouse IgG+IgA+IgM labeled with alkaline phosphatase, diluted in diluent was added to the wells and incubated

for 90 min at RT. Substrate was added and the plates were read at OD₄₀₅ on a Bio-Tek ELISA plate reader.

Blood Collection for Cell Isolations

Blood samples were collected from the coccygeal vein of healthy adult Holsteins, using acid citrate dextrose (10%) as the anticoagulant. Siliconized glassware was used for all cell isolations.

Polymorphonuclear Neutrophil Isolation

Neutrophils were obtained by the method of Carlson and Kaneko (1975). Whole blood was centrifuged ($1500 \times g$, 5 min, 4°C), and plasma, buffy coat, and one-third of the red blood cell pellet were removed. A double volume of ddH₂O was added to the cell suspension and mixed for 45 s to lyse the red blood cells. Isotonicity was restored by adding a single volume of 2.7% PBS. The suspension was centrifuged ($500 \times g$, 3 min, 4°C) and the pellet was washed two times in PBS. Total cell counts were determined using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL). Differential cell counts were determined microscopically using Wright-stained smears. Cell viability was determined using trypan blue exclusion.

Flow Cytometry Phagocytosis Assay

Phagocytosis was determined by flow cytometry according to Saad and Hageltorn (1985) with minor modifications. FITC-labeled Df was incubated with various sera or Hank's balanced salt solution for 30 min at 37°C with gentle rocking. Isolated neutrophils (10×10^6 /ml) were added, and the tubes were incubated for an additional 30 min at 37°C with gentle rocking. Phagocytosis was stopped by adding 1.0 ml of ice-cold 0.85% saline with 0.04% EDTA. The samples were analyzed by flow cytometry using an EPICS Profile flow cytometer (Coulter Electronics) equipped with a 488-nm argon ion laser. After gating on the appropriate cell population, the percentage of fluorescing cells was recorded. The ingested Df were differentiated from the adhered by quenching extracellular fluorescence with 400 μ l of 1% methylene blue.

Cell Culture

Mammary secretory epithelial cells isolated postmortem from a lactating cow were cultured on rat tail collagen-coated 60-mm dishes (Collaborative Biomedical Products, Bedford, MA) as described previously (Cifrian et al., 1994). The cells were cultured in growth medium containing 40% RPMI-1640 (JRH Biosciences, Lenexa,

KS), 40% DMEM (JRH Biosciences), 10% fetal bovine serum (JRH Biosciences), 2% antibiotic-antimycotic solution (Sigma Chemical Co.), 1 mM sodium pyruvate (Sigma Chemical Co.), 2 mM L-glutamine (JRH Biosciences), 40 mM HEPES buffer (Sigma Chemical Co.), bovine insulin (5 μ g/ml; Sigma Chemical Co.), hydrocortisone (1 μ g/ml; Sigma Chemical Co.), and bovine prolactin (1 μ g/ml; courtesy of D. J. Bolt, USDA, Animal Hormone Program, Beltsville, MD).

Growth of *Staphylococcus aureus*

Several colonies of Df from blood agar cultures were inoculated in 10 ml of trypticase soy broth. After incubation at 37°C overnight, 100- μ l aliquots were inoculated in 10 ml of fresh trypticase soy broth. After reaching the logarithmic phase (7 h), the organisms were harvested by centrifugation at $1500 \times g$ for 10 min. The cell pellet was resuspended in 3 ml of 1:1 ratio of RPMI-1640:DMEM (RM).

Adherence Assays

Secretory epithelial cells were plated on rat-tail collagen-coated 60-mm dishes (10^6 cells in 5 ml of growth medium) and grown to confluence by incubating at 37°C in 5% CO₂ for 7 d. The growth medium was replaced every 48 h. After reaching confluence, the growth medium was removed and 150 μ l of NaCl, pooled preimmunization or pooled immune sera, and 3 ml of sonicated Df in RM were added to the monolayers and incubated at 37°C in 5% CO₂ for 3 h with rocking. The unadhered Df were removed and the monolayers were washed 5 times by holding the dish at an angle and allowing 10 ml of PBS to flow over the monolayer. The monolayers were fixed with methanol and stained with Giemsa (Freshney, 1987). Adhered bacteria in 40 fields of 0.01 mm² were counted microscopically. The assay was performed in duplicate.

Statistical Analysis

Data were analyzed by mixed models analysis of variance techniques for repeated measures (SAS, version 8). Except for IgM, the fixed portion of the model included the effects of microsphere (Lys or Lys-S), treatment (FICA or NaCl), period (0, 2, 4, 7, 10, 13, 16, or 20 wk) plus two- and three-way interactions. For IgM group (Lys/FICA; Lys-S/FICA; and Lys-S/NaCl), period and the two-way interaction were included in the fixed portion of the model. The random portion of the model included the variation among cows within microsphere and treatment group and the variation within cows over periods (residual). The SAS mixed model procedure was

also used to model the correlations among repeated measures over time. The Schwarz's Bayesian goodness of fit statistic was used to choose a variance-covariance structure that adequately described the variances and covariances among the repeated measures. Normality of the model residuals were examined and found satisfactory.

RESULTS

Staphylococcus aureus Cell Wall Antibody Titers by Isotype

Cows receiving Lys/NaCl showed no increase in IgG₁ titers to cell wall over the course of the study (Figure 1; Table 1). Cows receiving Lys/FICA had the highest IgG₁ sera titers to cell wall. Maximal titers occurred at 4 wk, declined to 10 wk then rose to 13 wk and remained level to 20 wk ($P < 0.05$ at 2, 4, and 13 to 20 wk). Similarly, IgG₁ titers of cows receiving Lys-S/NaCl increased at 2 wk, declined to 4 wk, then rose again to 16 wk and plateaued to 20 wk ($P < 0.05$ at 2 and 13 to 20 wk). Cows receiving Lys-S/FICA had maximal IgG₁ titers at 2 wk, which declined to 10 wk then peaked again at 13 wk. Titers declined to 16 wk then plateaued to 20 wk.

The IgG₂ titers for cows receiving Lys/NaCl increased slightly at 4 wk but returned to preimmunization levels by 10 wk. Cows receiving Lys/FICA had maximal IgG₂ titers to cell wall antigens at 4 wk and peaked again at 13 wk before declining rapidly at 16 wk ($P < 0.05$ at 2 to 20 wk). Similarly, the IgG₂ sera titers for cows receiving Lys-S/NaCl peaked at 4 wk then remained level until 13 wk before declining at 16 wk ($P < 0.05$ at 5 to 13 and 20 wk). The Lys-S/FICA group had peak IgG₂ sera titers at 2 wk, decreased at 7 wk, then rose again until 10 wk and declined at 16 wk ($P < 0.05$ at 2, 4, and 10 to 20 wk). All groups plateaued from 16 to 20 wk.

Only the Lys/FICA group showed an increase in IgM antibody titer to cell wall (Figure 1). Peak titers occurred at 2 wk and again at 20 wk ($P < 0.05$ at 2 and 20 wk).

Staphylococcus aureus Capsule Antibody Titers by Isotype

IgG₁ sera titers to capsule of cows receiving Lys/FICA peaked at 7 wk, declined at 10 wk, and increased significantly at 13 wk ($P < 0.05$ at 7 and 13 to 20 wk) (Figure 2). Cows in the Lys-S/NaCl group had peak titers at 7 wk and was significantly elevated from 13 to 20 wk ($P < 0.05$ at 7 to 20 wk). Serum titers of the Lys-S/FICA group were significantly elevated at 7, 13, and 20 wk ($P < 0.05$ at 7, 13, and 20 wk).

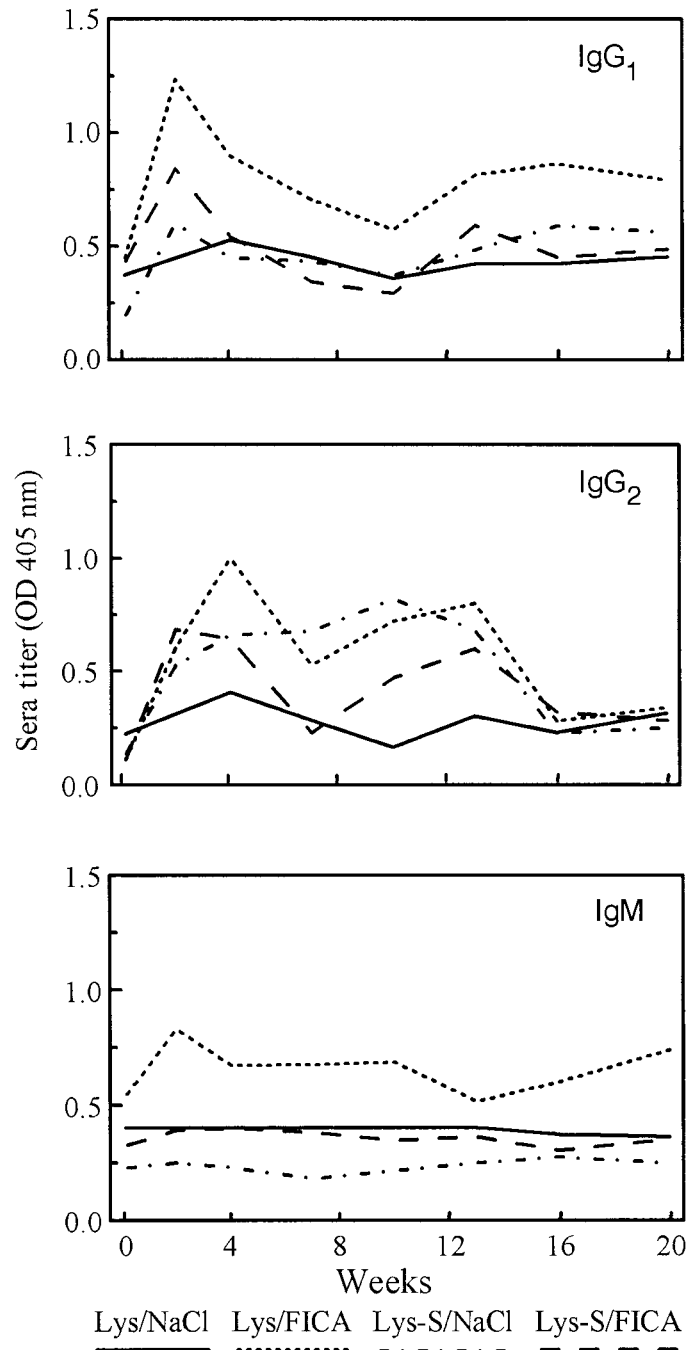


Figure 1. Anti-*Staphylococcus aureus* Smith Compact (Cp) cell wall antibodies by isotype. Lys/NaCl = Cows immunized with 500 μ g of *S. aureus* Df lysate (Df lysate) in NaCl. Lys/FICA = 500 μ g of Df lysate emulsified in FICA. Lys-S/NaCl = 500 μ g of Df lysate encapsulated in microspheres (<10 and >10 μ m) suspended in NaCl. Lys-S/FICA = 500 μ g of Df lysate encapsulated in microspheres emulsified in Freund's incomplete adjuvant.

Table 1. Antibody isotype titers before immunization and at wk 20.

Antigen	Treatment ¹	Antibody isotype OD at 0 and 20 wk					
		IgG ₁		IgG ₂		IgM	
		0	20	0	20	0	20
Cell wall	Lys/NaCl	0.37	0.45	0.22	0.31	0.40	0.36
	Lys/FICA	0.44	0.78*	0.09	0.33*	0.53	0.74*
	Lys-S/NaCl	0.19	0.56*	0.12	0.24*	0.23	0.24
	Lys-S/FICA	0.42	0.48	0.08	0.27*	0.32	0.35
Capsule	Lys/NaCl	0.55	0.53	0.58	0.43	0.47	0.49
	Lys/FICA	0.51	0.93*	0.26	0.57*	0.46	0.59*
	Lys-S/NaCl	0.39	0.75*	0.14	0.43*	0.40	0.55
	Lys-S/FICA	0.38	1.01*	0.03	0.60*	0.50	0.52

¹Lys/NaCl = immunized with Df lysate in NaCl. Lys/FICA = immunized with Df lysate emulsified in FICA. Lys-S/NaCl = immunized with Df lysate in microspheres suspended in NaCl. Lys-S/FICA = immunized with Df lysate in microspheres emulsified in FICA.

*OD means at 20 wk are significantly different ($P < 0.05$) from 0 wk.

The IgG₂ titers of the Lys/FICA group peaked at 7 wk, declined at 16 wk, but rose again at 20 wk ($P < 0.05$ at 7 to 13 and 20 wk). The Lys-S/NaCl group IgG₂ titers increased at 7 wk and tended to remain above preimmunization to 20 wk ($P < 0.05$ at 10 and 20 wk). The IgG₂ titers of cows receiving Lys-S/FICA increased significantly at 2 wk and remained significantly higher than preimmunization to 20 wk ($P < 0.05$).

The IgM titers increased significantly at 7 wk for cows receiving Lys/FICA and at 20 wk and for cows receiving Lys-S/NaCl ($P < 0.05$).

Neutrophil Phagocytosis

Neutrophil phagocytosis of Df opsonized with pooled sera was determined at 0 wk (preimmunization), 7 wk (high capsule sera titer), 20 wk, and at 52 wk (Figure 3). Before immunization, the Lys/NaCl cows had significantly ($P < 0.05$) higher percent phagocytosis of Df than either of the other groups. From 0 to 7 wk, there was a significant decrease ($P < 0.05$) in percent phagocytosis with sera from the Lys/NaCl, Lys-S/NaCl, and Lys-S/FICA groups. Sera from the Lys/FICA group significantly increased ($P < 0.001$) phagocytosis from 0 to 7 wk then decreased phagocytosis at 20 wk ($P < 0.001$). At 20 wk, the Lys/NaCl and Lys-S/FICA groups significantly increased ($P < 0.05$) phagocytosis compared with 0 wk, with the Lys-S/FICA group having the highest percentage of phagocytosis. At 20 wk, cows receiving Lys-S/NaCl remained unchanged from 7 wk. Only sera from the Lys/FICA, Lys-S/NaCl, and Lys-S/FICA groups were available for phagocytosis at 52 wk. Phagocytosis with sera from Lys/FICA decreased from 20 wk but remained higher than preimmunization. At 52 wk the ability of sera from cows receiving Lys-S/NaCl to support phagocytosis decreased from 20 wk and was significantly lower ($P < 0.05$) than preimmunization.

Phagocytosis with sera from the Lys-S/FICA group decreased from 20 wk but was significantly greater ($P < 0.001$) than preimmunization sera or 52 wk sera from the Lys/FICA or Lys-S/NaCl groups.

Staphylococcus aureus Adherence

The ability of pooled sera to prevent adherence of Df to cultured bovine mammary epithelial cells is shown in Table 2. Incubation of Df with preimmunization sera significantly decreased ($P < 0.01$) adherence compared with Df incubated in NaCl. A further decrease in adherence was observed when Df was incubated with pooled immune sera (20 wk) from the Lys/NaCl and Lys-S/NaCl groups. However, the greatest decrease in adherence was observed when Df was incubated with pooled immune sera from Lys/FICA and Lys-S/FICA groups.

DISCUSSION

The major virulence mechanisms of *S. aureus* are their ability to evade neutrophil phagocytosis by production of an exopolysaccharide capsule and to adhere to and penetrate mammary tissue. To combat these virulence mechanisms, a successful vaccine against *S. aureus* would require long-term production of antibodies that are opsonic for neutrophils and prevent adherence. Conventional vaccines use adjuvants and multiple boosts to promote long-term antibody production. Controlled release mechanisms such as poly(lactide-co-glycolide) microspheres serve as alternatives to conventional vaccines by initiating an immune response and providing a long-term supply of antigen with a single injection. Small microspheres ($<10 \mu\text{m}$) are taken up by antigen-presenting cells, and the 50:50 lactide:glycolide ratio rapidly degrade (O'Brien et al., 2000) but present sufficient antigen to stimulate the strong initial anti-

body response. The larger microspheres ($>10\ \mu\text{m}$; 75:25 lactide:glycolide ratio) preclude their uptake by antigen-presenting cells, thus serving as an antigen depot that slowly release their antigen over an extended pe-

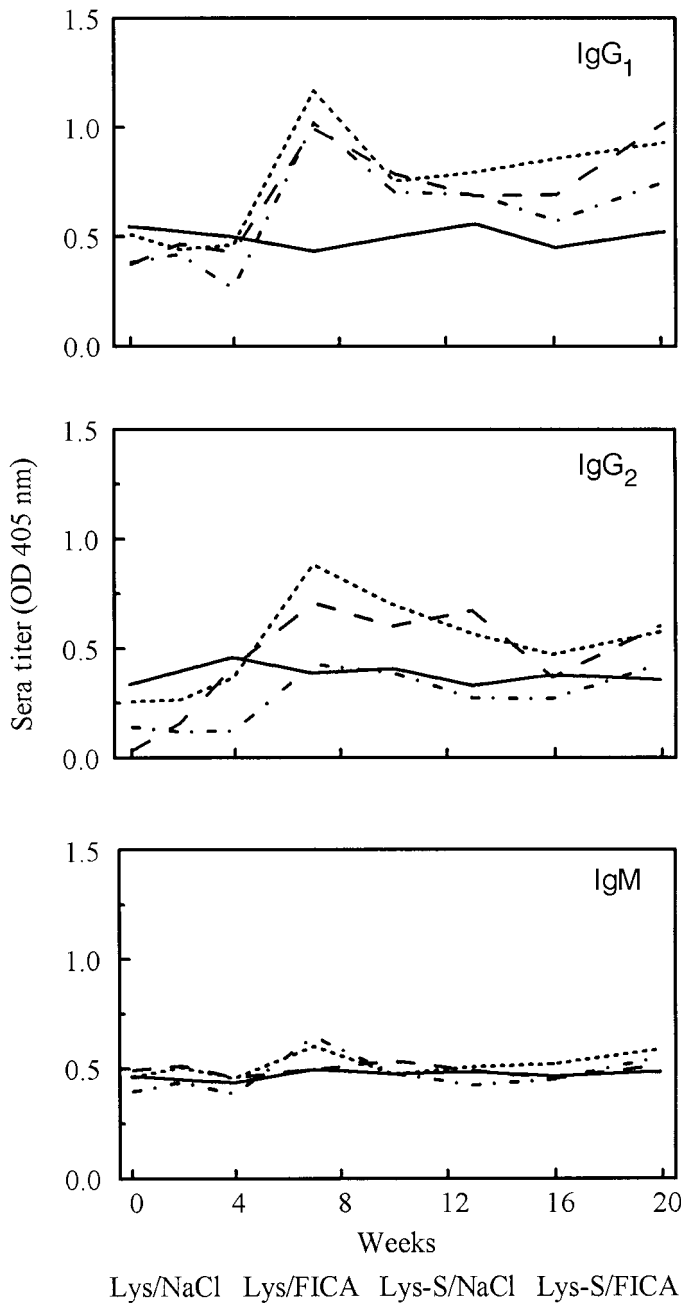


Figure 2. Anti-*Staphylococcus aureus* Smith Diffuse (Df) capsule antibodies by isotype. Lys/N aCl = Cows immunized with 500 μg of *S. aureus* Df lysate (Df lysate) in NaCl. Lys/FICA = 500 μg of Df lysate emulsified in Freund's incomplete adjuvant (FICA). Lys-S/N aCl = 500 μg of Df lysate encapsulated in microspheres (<10 and $>10\ \mu\text{m}$) suspended in NaCl. Lys-S/FICA = 500 μg of Df lysate encapsulated in microspheres emulsified in FICA.

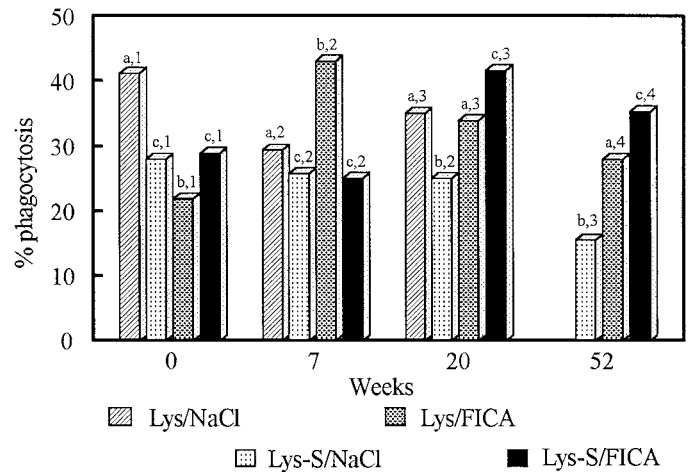


Figure 3. Neutrophil phagocytosis of *Staphylococcus aureus* Smith Diffuse (Df) opsonized with pooled preimmune and immune sera. Lys/N aCl = Cows immunized with 500 μg of *S. aureus* Df lysate (Df lysate) in NaCl. Lys/FICA = 500 μg of Df lysate emulsified in FICA. Lys-S/N aCl = 500 μg of Df lysate encapsulated in microspheres (<10 and $>10\ \mu\text{m}$) suspended in NaCl. Lys-S/FICA = 500 μg of Df lysate encapsulated in microspheres emulsified in FICA. ^{a,b,c}Means within a sampling period without common letters differ ($P < 0.05$). ^{1,2,3,4}Means within a group without common numbers differ ($P < 0.05$).

riod of time. In the current study, a single injection of *S. aureus* lysate, free or encapsulated in microspheres, with and without emulsification with FICA, was tested for its ability to produce a long-term antibody response.

The failure of cows immunized with Lys/N aCl to produce an antibody response to cell wall or capsule is indicative of the need for adequate antigen presentation. The high serum antibody titers to cell wall and capsule in cows immunized with *S. aureus* Lys/FICA indicate the effectiveness of early antigen presentation and persistence. The delayed antibody response to capsule compared with cell wall in all treatments could be attributed to its low immunogenicity. The decrease in serum IgG₁ and IgG₂ titers to cell wall at wk 7 and 10 followed by a rise at wk 13 may have been due to transport of Ig into the mammary gland prior to parturition. The decreased IgG₁ and IgG₂ response to cell wall and the lack of an IgM response to Lys-S/N aCl and Lys-S/FICA were attributed to the slow release of antigen incorporated in the microspheres.

Serum from cows receiving Lys/FICA had the highest percentage phagocytosis at 7 wk, which corresponds to peak IgG₂ antibody titer to capsule. However, serum from Lys-S/FICA was the most effective in promoting neutrophil phagocytosis at 20 and 52 wk. Phagocytosis increased from 7 to 20 wk and remained elevated at 52 wk. The delayed response observed with Lys-S/FICA compared with Lys/FICA was attributed to the delay in antibody response and possibly to an increase in

Table 2. Adherence of *Staphylococcus aureus* Smith diffuse (Df) to bovine mammary epithelial cells after incubation with pooled preimmunization and immune sera (20 wk). Values are mean number of bacteria counted in 40 fields.

Saline	Serum ¹				
	Preimmune	Lys/NaCl	Lys/FICA	Lys-S/NaCl	Lys-S/FICA
26.0 ^a	10.5 ^b	4.5 ^c	0.5 ^d	6.0 ^c	1.0 ^d

^{a,b,c,d}Values without common superscripts are significantly different ($P < 0.01$).

¹Lys/NaCl = immunized with Df lysate in NaCl. Lys/FICA = immunized with Df lysate emulsified in FICA. Lys-S/NaCl = immunized with Df lysate in microspheres suspended in NaCl. Lys-S/FICA = immunized with Df lysate in microspheres emulsified in FICA.

antibody affinity, known to occur using microspheres as an antigen delivery system. The sustained level of phagocytosis using microspheres was also obtained in a previous study using *S. aureus* capsule conjugated to *Pseudomonas aeruginosa* encapsulated in microspheres and emulsified in FICA (O'Brien et al., 2000).

Because adherence is the first step in establishing a chronic bacterial infection, a vaccine that would prevent adherence would greatly aid in the prevention of chronic *S. aureus* infections in the bovine mammary gland. This would also give neutrophils in lacteal secretions additional opportunity to phagocytose the organisms, thus facilitating flushing of the organisms from the gland during milking. Sera from cows receiving Lys/FICA and Lys-S/FICA were the most effective in preventing adherence of Df to bovine mammary epithelial cells. The high IgG₁ and IgG₂ titers at 20 wk could account for the inhibition of Df adherence to mammary epithelium. Cifrian et al. (1994) showed that *S. aureus* Smith Df is less adherent to mammary epithelial cells than the unencapsulated Smith Cp. However, it is known that during log phase, when these organisms were used in the adherence assays, Smith Df has areas of exposed cell walls (Guidry et al., 1991). Therefore, it is important to have antibodies to capsule and cell wall components to prevent adherence. The decrease in adherence using preimmune serum could be attributed to antibodies present due to prior exposure to *S. aureus* antigens or the nonspecific blockage by other serum components.

Similarity of the results between Lys/FICA and Lys-S/FICA suggests that these protocols are of equal value. However, there are several distinct advantages associated with the microspheres: 1) antigen release can be controlled by the ratio of lactic acid:glycolic acid and the size of the microspheres (O'Brien et al., 1996); 2) control of antigen presentation to or exclusion from given cell types; 3) sustained presentation of antigen over long periods of time with a single injection; and 4) sustained neutrophil phagocytosis.

These data show that FICA is needed to obtain an effective, sustained immune response to *S. aureus* cap-

sule. The data also demonstrate that *S. aureus* antigen encapsulated in microspheres and emulsified in FICA results in the long-term production of antibodies that are opsonic to for neutrophils and block adherence of *S. aureus* to mammary epithelium.

CONCLUSIONS

Antigen in microspheres produced a similar antibody response to antigen emulsified in FICA but of a lesser magnitude. However, antibodies produced by antigen in microspheres were more opsonic for bovine neutrophils and as inhibitory to adherence of *S. aureus* to mammary epithelium as antigens in FICA.

The nontoxic nature of the microspheres, the ability to control antigen release and presentation, and the benefit of a single injection has application to large herds and beef cattle and warrant additional studies.

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